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Correlation of Fibrosis and Transforming Growth Factor- β Type 2 Levels in the Eye

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Abstract

Approximately 1 out of every 10 eyes undergoing surgery for retinal detachment develops excessive intraocular fibrosis that can lead to traction retinal detachment and ultimate blindness. This disease process has been termed proliferative vitreoretinopathy (PVR). The ability to monitor and grade this fibrotic response accurately within the eye as well as the ability to aspirate vitreous cavity fluid bathing the fibrotic tissue makes this an ideal setting in which to investigate the development of fibrosis. Although laboratory studies have recently shown that transforming growth factor- β (TGF- β) can enhance fibrosis, little clinical evidence is yet available correlating the level of this or other growth factors with the degree of fibrosis in a clinical setting. We have found that vitreous aspirates from eyes with intraocular fibrosis associated with PVR have more than three times the amount of TGF- β ($1,200 \pm 300$ pM [SEM]) found in eyes with uncomplicated retinal detachments without intraocular fibrosis (360 ± 91 pM [SEM]). Using an *in vitro* assay, 84–100% of the TGF- β activity could be blocked with specific antibodies against TGF- β_2 , whereas only 10–21% could be blocked by specific antibodies against TGF- β_1 . TGF- β_1 was used in an animal model of traction retinal detachment. Since β_1 and β_2 have essentially identical biologic effects and only human β_1 was available in quantities required, β_1 was chosen for these *in vivo* studies. The injection of TGF- β_1 plus fibronectin (FN) but not TGF- β_1 alone into the vitreous cavity of rabbits resulted in the increased formation of intraocular fibrosis and traction retinal detachments as compared to control eyes. In previous studies, intravitreal FN levels were also found to be elevated in eyes with intraocular fibrosis.

Introduction

The role of peptide growth factors in wound healing and fibrosis has recently received particular attention. Platelet-derived growth factor (PDGF),¹ fibroblast growth factor (FGF),

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Abbreviations used in this paper: FGF, fibroblast growth factor; FN, fibronectin; PDGF, platelet-derived growth factor; PVR, proliferative vitreoretinopathy; TGF- β , transforming growth factor- β .

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and transforming growth factor- β (TGF- β) have recently been shown to have potential roles in the fibrotic process. PDGF is released from the α -granules of platelets, as well as from monocytes, and is a chemoattractant and a mitogen for fibroblasts (1). FGF is also a mitogen for fibroblasts and induces an increase in DNA content when introduced into porous subcutaneous chambers (2). TGF- β appears to have a particularly important role in the fibrotic process. This peptide is found in high concentrations in the α -granules of platelets (3) and is also secreted by activated T lymphocytes (4) and macrophages (5). Like PDGF, it is also chemotactic for both monocytes (6) and fibroblasts (7). When injected subcutaneously in newborn mice, it causes a rapid fibrotic and angiogenic response at the site of injection (8); the new tissue formed is essentially granulation tissue (8, 9). *In vitro*, TGF- β has been shown to regulate both synthesis (8, 9) and degradation of matrix proteins, leading to their increased accumulation (10). TGF- β has recently been found to exist in two distinct molecular forms, TGF- β_1 and TGF- β_2 (10, 11). Current data suggest that there may be separate receptors for TGF- β_1 and TGF- β_2 , some of which are cross-reactive (10, 11). However, the relative roles of TGF- β_1 and TGF- β_2 in the fibrotic process have not yet been determined.

Although laboratory studies have shown that both PDGF and TGF- β can enhance fibrosis, little clinical evidence is yet available correlating the levels of these growth factors with the degree of fibrosis in a clinical setting. An important recent study has shown that PDGF is secreted in exaggerated amounts by alveolar macrophages from patients with idiopathic pulmonary fibrosis (12), demonstrating a possible association between a peptide growth factor and a disease process involving pathologic fibrosis, but the actual levels within the fibrotic pulmonary tissue were not determined. Proliferative vitreoretinopathy (PVR), the most common cause of failure in retinal reattachment surgery, is an ocular disorder characterized by excessive fibrosis on both surfaces of the retina and within the vitreous cavity (13–17). The intraocular fibrous tissue is composed of retinal pigment epithelial cells, glial cells, fibroblasts, and macrophages as well as an extensive accumulation of extracellular matrix proteins (13–17). The fibrosis results in the development of contractile forces on the retina causing retinal folding and traction retinal detachments. The eye provides a unique window to observe this fibrotic process, allowing assessment of both its extent and severity.

Several studies have suggested that retinal pigment epithelial cells play a central role in the development of PVR (13, 18–21). Recently, we have found that cultures of human retinal pigment epithelial cells can synthesize and release significant amounts of TGF- β (Connor, T. B., A. B. Roberts, M. B. Sporn, and B. M. Glaser, manuscript in preparation), thus providing a possible link between TGF- β and the fibrotic process of PVR. Therefore, to determine if TGF- β might play a role in the fibrosis occurring in PVR, intraocular fluid speci-

mens from patients with varying degrees of intraocular fibrosis were analyzed for the presence of TGF- β . We now report that specimens from eyes with intraocular fibrosis associated with PVR have elevated levels of TGF- β when compared to specimens from eyes with uncomplicated retinal detachments without fibrosis and that these levels correlate with the degree of intraocular fibrosis. Furthermore, TGF- β_1 was the predominant form present in the intraocular fluid studied.

Methods

Study population

Intraocular fluid specimens were obtained from eyes with PVR and with uncomplicated retinal detachments. The diagnosis of PVR was established in 35 patients (10 women and 25 men, ages 5–78 yr, mean age 46 yr) by preoperative and intraoperative clinical examination. The diagnosis of uncomplicated retinal detachment was established in nine patients (four women and five men, ages 29–68 yr, mean age 57 yr) by clinical examination. The severity of PVR was graded in a masked fashion using the system proposed by the Retina Society Terminology Committee (22) and grouped in the following manner according to severity: mild, grade C1 or less; moderate, grades C2–C3; and severe, grades D1–D3.

Intraocular fluid samples

Vitreous aspirates (0.5–1.73 ml) were obtained via the pars plana from eyes with PVR before vitrectomy using a 30-gauge needle on a 1-ml syringe, transferred to a sterile tube, and immediately stored at -70°C. Vitreous aspirates were similarly obtained from eyes with shallow uncomplicated retinal detachments, in which subretinal fluid drainage could not be adequately performed, and which required aspiration of liquid vitreous to provide a needed decrease in intraocular volume to accommodate an encircling buckle. In all cases, intraocular fluid was aspirated before starting any intraocular infusion. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

Quantification of TGF- β

As has been found for TGF- β secreted into medium by cells (23, 24), as well as TGF- β released from platelets (25), and in wound fluid (26), ~87% of the TGF- β in intraocular fluid was latent. The degree of latency was not a function of the disease state of the patients in this study. For this reason, samples were activated by acidification with 150 mM HCl for 30 min followed by reneutralization prior to assay (24). A competitive radioreceptor binding assay using A549 human lung carcinoma cells (24) and an assay measuring formation of colonies of NRK cells in soft agar in the presence of epidermal growth factor (27) were carried out exactly as previously described. An assay of the inhibition by TGF- β of the growth of CCL64 mink lung epithelial cells (28, 29) was modified as follows: cells were seeded into 24-well multidishes at a density of 5×10^4 cells per well in 0.5 ml of 0.2% fetal calf serum in Dulbecco's modified Eagle's medium. 1 h later, TGF- β or samples of intraocular fluid (or antibodies, when appropriate) were added and the incubation continued for 22 h. [125 I]iododeoxyuridine or [3 H]-thymidine (Amersham Corp., Arlington Heights, IL; 0.5 μ Ci/well) was then added for an additional 2-h incubation. After fixation in methanol/acetic acid (3:1) and washing, cells were dissolved in 1 N NaOH and counted in a gamma or liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Antibodies to TGF- β_1 and TGF- β_2

Turkeys were injected with uncoupled porcine TGF- β_1 or TGF- β_2 (100 μ g) in Freund's complete adjuvant and boosted every 2 wk with equivalent amounts of TGF- β in incomplete Freund's adjuvant. Antisera were titrated in an ELISA and measured for blocking activity in either a radioreceptor binding assay or the growth inhibition assay described above.

TGF- β_1 was purified from human platelets as previously described (33), followed by a final purification step using high-performance liquid chromatography. The peptide was quantitated by amino acid analysis. TGF- β_1 from porcine platelets (11) was purchased from R&D Systems, Minneapolis, MN.

Effect of intravitreal injection of TGF- β_1 on intraocular fibrosis

Pigmented Dutch belted rabbits weighing 5–8 lb (2.3–3.6 kg) were anesthetized by intramuscular injection of ketamine and xylazine. The pupils were dilated with 1% tropicamide and one eye was gently prolapsed and draped. An 8-ml transcleral incision was made 1–2 mm posterior to the limbus. The wound was closed with multiple interrupted 8-0 black silk sutures. After wound closure, 0.2 ml of one of the following solutions was injected into the midvitreous cavity via a 30-gauge needle 1–2 mm posterior to the limbus in the superotemporal quadrant. The following solutions were injected:

Control. Eagle's minimum essential medium with 0.1 mg/ml BSA.

Group 1. Eagle's minimum essential medium with 0.1 mg/ml BSA and 100 ng of TGF- β_1 .

Group 2. Eagle's minimum essential medium with 0.1 mg/ml BSA, 100 ng of TGF- β_1 , and 50 μ g of human fibronectin (FN).

Group 3. Eagle's minimum essential medium with 0.1 mg/ml BSA and 50 μ g of FN.

The same injections were repeated after 24 h. The rabbits were examined postoperatively at 24 h and thereafter at weekly intervals. Intravitreal fibrosis was judged by monitoring the development of traction retinal detachment.

Statistical analysis

Samples were sorted according to disease type and severity as outlined above: retinal detachment, mild PVR, moderate PVR, and severe PVR. The mean of duplicate measurements of each sample was used for calculations. The Kruskal-Wallis test (30), a nonparametric one-way analysis of variance, was used to determine whether these groups differed in regard to their TGF- β levels. The Wilcoxon rank sum test (30) was used to compare each disease category individually with each of the other categories.

Results

The concentration of total TGF- β -like activity in the vitreous aspirates was determined using the competitive radioreceptor binding assay and was calculated by comparison of dilution curves of aspirates with that of a standard curve generated using purified TGF- β_1 (Fig. 1 and Table I). Vitreous aspirates from eyes with PVR had more than three times the amount of total TGF- β ($1,200 \pm 300$ pM [SEM]) as that found in eyes with uncomplicated retinal detachments (360 ± 90 pM [SEM]) (Fig. 2). These levels represent the sum of both intrinsically active and latent TGF- β , since samples were acidified before assay, a treatment known to activate latent TGF- β (23, 24). Vitreous aspirates assayed without acid pretreatment had 9–13% of the activity found following acid treatment. A comparable degree of enhancement of TGF- β activity has been observed for medium conditioned by many different human and rodent cell lines (23, 24). The degree of enhancement following acid activation of vitreous aspirates did not correlate with severity nor type of disease. Protein concentrations of the vitreous aspirates were elevated in eyes with PVR (16.9 ± 3.3 mg/ml [SEM]) when compared to eyes with uncomplicated retinal detachment (2.7 ± 0.7 mg/ml [SEM]) (see Table I).

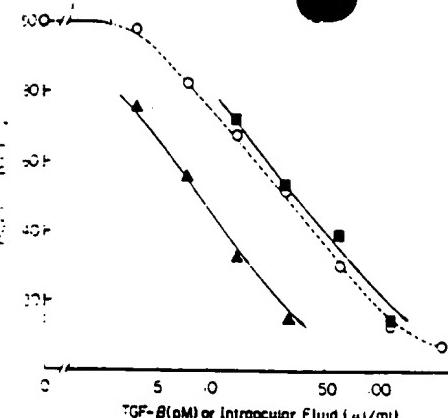


Figure 1. Quantitation of the TGF- β levels of intraocular fluid samples by a competitive radioreceptor binding assay. Acid activated (see Methods) intraocular fluid samples were assayed for their ability to compete with [125 I]-TGF- β_1 for binding to A549 human lung carcinoma cells. The concentration of TGF- β -like activity in the patient samples was calculated by comparison of the sample dilution curves with that of the TGF- β_1 standard (○). Patient 2 (■) was diagnosed as having mild PVR, while in patient 6 (▲) the disease was moderate.

Samples from eyes with PVR were grouped according to disease severity as described in the Methods section. Analysis of TGF- β levels in these groups revealed that as clinical disease progressed from mild to moderate to severe, total TGF- β levels likewise increased (Fig. 3). Total TGF- β levels of these four groups were analyzed collectively with the Kruskal-Wallis nonparametric one-way analysis of variance which showed that the groups are different ($P < 0.001$), with the mean ranks of TGF- β levels of each group increasing monotonically, paralleling the increase in clinical severity seen among the four groups (Table II). Total TGF- β levels of the groups were then analyzed independently with each of the other groups using the Wilcoxon rank sum test. This analysis demonstrated that total TGF- β levels of the group with severe PVR are different from all other groups ($P < 0.001$), and the group with moderate PVR is different from the group with uncomplicated RD ($P < 0.02$). These data demonstrate that as the severity of PVR increased, intraocular levels of TGF- β from these same eyes likewise increased.

TGF-beta is also a potent inhibitor of the growth of CCL 64 cells (28, 29). As further confirmation of the levels of total TGF- β detected by the radioreceptor binding assay, several intraocular fluid samples were tested for growth inhibition of CCL 64 cells and colony formation of NRK cells in soft agar. Values of total TGF- β in the specimens as determined by the radioreceptor binding assay correlated with values determined by these assays (Fig. 4A). This finding confirms the presence of TGF- β in the intraocular fluid specimens, and it also demonstrates that the samples contain no significant concentrations of mitogens which would oppose the inhibitory action of TGF- β in the growth inhibition assay.

In order to determine the type of TGF- β in the vitreous aspirates, type-specific polyclonal antibodies raised in turkeys against either porcine TGF- β_1 or TGF- β_2 were used in an attempt to block the growth inhibitory activity of the samples. As shown in Fig. 4B, antibodies raised against TGF- β_1 specifically blocked the activity of added TGF- β_1 in the assay, whereas antibodies raised against TGF- β_2 blocked the activity

Table I. Patient Data

Patient	Age	Sex	Diagnosis	Protein	TGF- β
				mg/ml	pM
1	53	M	Moderate PVR	30.0	1,000
2	60	M	Mild PVR	3.1	490
3	29	M	RD	0.5	52
4	41	M	Mild PVR	1.4	60
5	68	F	RD	1.3	210
6	39	F	Moderate PVR	17.0	700
7	58	F	RD	2.3	280
8	33	F	Severe PVR	32.0	1,900
9	73	M	Severe PVR	30.0	1,800
10	68	M	Severe PVR	10.0	940
11	56	F	RD	6.2	960
12	17	M	Moderate PVR	6.2	840
13	35	M	Severe PVR	26.0	990
14	69	M	Severe PVR	18.0	1,500
15	78	F	Mild PVR	4.7	250
16	31	M	Mild PVR	5.8	540
17	39	M	Severe PVR	56.0	1,300
18	47	M	Moderate PVR	4.0	460
19	44	M	Severe PVR	29.0	1,000
20	60	M	Mild PVR	1.3	310
21	62	M	RD	1.7	120
22	5	F	Severe PVR	88.0	1,800
23	64	F	Acute RD	1.6	380
24	57	M	Moderate PVR	2.4	410
25	51	M	Mild PVR	11.5	850
26	47	M	Acute RD	2.3	340
27	47	F	Moderate PVR	9.8	560
28	47	M	Mild PVR	2.7	580
29	20	M	Severe PVR	23.0	1,300
30	17	F	Severe PVR	5.4	840
31	13	M	Moderate PVR	5.8	890
32	72	M	Acute RD	5.5	600
33	78	F	Mild PVR	5.4	860
34	60	M	Mild PVR	8.1	930
35	32	M	Severe PVR	30.1	1,300
36	59	M	Severe PVR	22.0	1,900
37	60	M	Moderate PVR	5.5	560
38	61	F	Severe PVR	23.0	940
39	42	M	Severe PVR	4.5	470
40	26	F	Mild PVR	Not done	560
41	57	M	RD	Not done	2,300
42	42	F	Severe PVR	Not done	2,300
43	66	M	Severe PVR	Not done	11,200
44	41	M	Moderate PVR	Not done	560

Diagnosis: PVR, mild, moderate, or severe; RD, retinal detachment, uncomplicated.

Protein: determined by the bicinchoninic assay (Pierce Chemical Co.).

of added TGF- β_2 and did not block the activity of added TGF- β_1 . Antibodies against TGF- β_2 blocked 84–100% of the growth inhibitory activity of the vitreous aspirates, whereas antibodies against TGF- β_1 blocked ~10–21% (see Fig. 4B). These findings not only serve to further confirm the presence of TGF- β in the vitreous aspirates, this time by immunological criteria, but also provide evidence about the origin of the

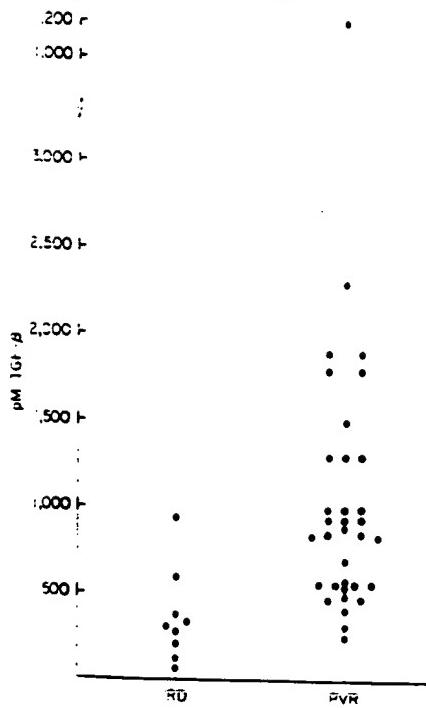


Figure 2. Quantitation of the TGF- β levels of intraocular fluid samples by competitive radioreceptor assay, as described in Fig. 1, grouped according to the presence or absence of PVR.

TGF- β found in intraocular fluid. TGF- β_2 is not found in human platelets or serum as is TGF- β_1 (11), thus suggesting that the source of TGF- β in the vitreous is not from serous

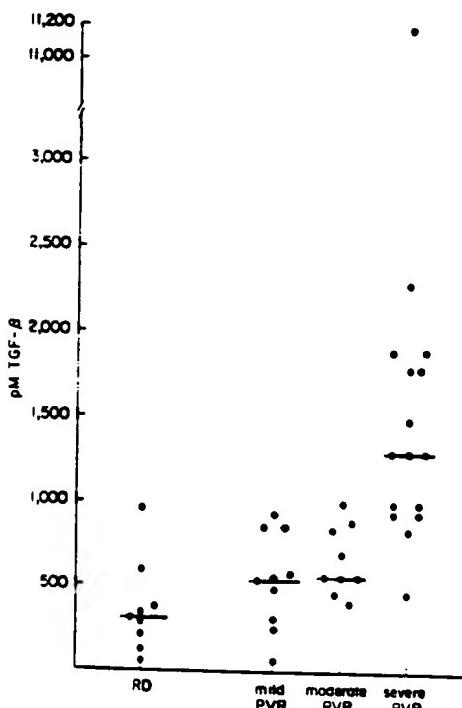


Figure 3. Quantitation of the TGF- β levels of intraocular fluid samples by competitive radioreceptor assay, as described in Fig. 1, grouped according to severity of PVR. Horizontal lines indicate median values.

Table II. TGF- β Levels by Diagnosis Group

Group	No. of samples	Mean TGF- β	Mean rank*
Retinal detachment	9	360	10.28
Mild PVR	10	543	16.00
Moderate PVR†	9	664	20.17
Severe PVR‡	16	1968	34.75

* $P < 0.001$. Kruskal-Wallis one-way analysis of variance.

† Moderate PVR group different from RD group, $P < 0.02$ (Wilcoxon rank sum test).

‡ Severe PVR group different from all other groups, $P < 0.001$ (Wilcoxon rank sum test).

exudation but may in fact be synthesized endogenously within the eye. In only one case, patient number 43, was there a predominance of TGF- β_1 over TGF- β_2 (data not shown). This patient had extraordinarily severe PVR associated with the highest recorded level of total TGF- β in our series and may have suffered an unusually severe degree of breakdown of the blood-ocular barriers.

The ability of TGF- β_1 to induce intravitreal fibrosis was determined by monitoring the formation of traction retinal detachment following intravitreal injection in an animal model. Traction retinal detachment occurred in only 1 out of 12 control eyes. 6 wk after the intravitreal injection of TGF- β_1 , traction retinal detachments were found in only 4 of 10 eyes ($P = 0.135$, Fisher's exact test). However, 6 wk after the injection of TGF- β_1 plus FN, 10 of 11 eyes developed traction retinal detachments ($P = 0.0001$). The injection of FN alone resulted in the development of a traction retinal detachment in only 1 of 12 eyes ($P = 1.0$).

Discussion

Approximately 1 out of every 10 eyes undergoing surgery for a retinal detachment develops excessive intraocular fibrosis that can lead to traction retinal detachment and ultimate blindness. The ability to monitor this intraocular fibrotic response both visually and photographically, the ability to use the degree of retinal detachment and contraction as an estimate of the magnitude of this fibrosis, plus the fact that fluid can be readily aspirated from the region directly bathing the fibrotic tissue at the time of surgery makes this an ideal situation in which to investigate the intrinsic role of assayable factors in the development of fibrosis in a clinical setting. In addition, the ability to see the entire area of involved tissue avoids random sampling errors that may occur during biopsy as required for the examination of fibrosis in other tissues. We now show, for the first time, that the level of total TGF- β within fluids bathing developing fibrotic tissue correlates with the degree of fibrosis, using three independent methods of detection including a radioreceptor binding assay, an assay of bioactivity, and an immunologic assay. Furthermore, using specific antibodies against TGF- β_1 and TGF- β_2 , we have found that the majority of the immunoreactive TGF- β is TGF- β_2 ; This is the first time that any tissue has been found to have a greater amount of TGF- β_2 compared to TGF- β_1 .

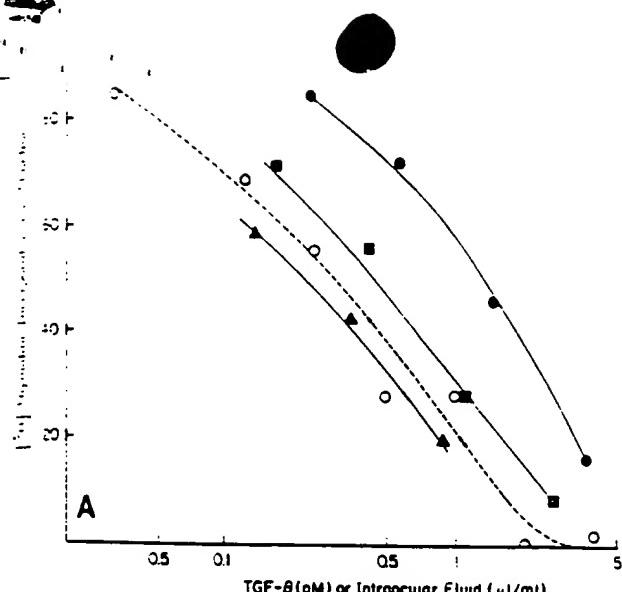
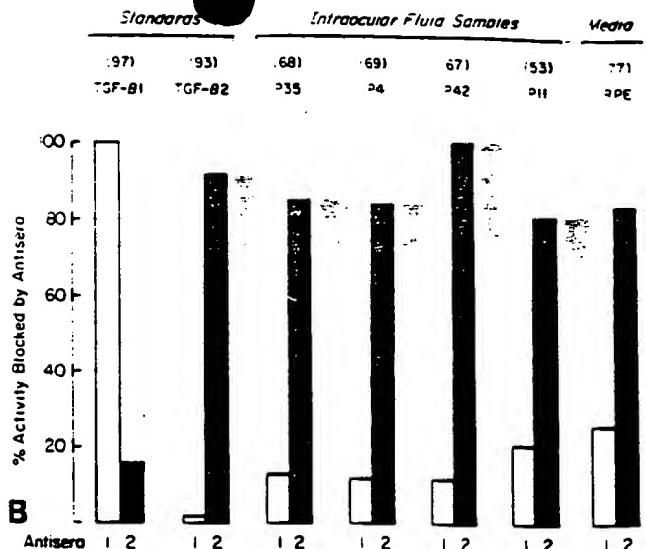


Figure 4. Antibodies to porcine TGF- β_2 block the ability of intraocular fluid to inhibit the growth of CCL64 cells. (A) Dose-response curves of the growth inhibition of human TGF- β_1 (○) and acid-activated samples of intraocular fluid: patient 26, retinal detachment (●); patient 2, mild PVR (▲); patient 9, severe PVR (△). Based on this assay, the concentration of TGF- β -like activity in the patient samples was estimated to be 140, 380, and 600 pM for samples 26, 2, and 9, respectively. (B) Acid-activated samples of intraocular fluid, medium conditioned by human RPE cells, human TGF- β_1 , or porcine TGF- β_2 , were assayed in the presence of either control antisera, antisera raised against TGF- β_1 (open bars), or antisera raised against TGF- β_2 (solid bars); as described in the Methods section. The antisera were used at 1:1,000 dilution. The percentage of the activity blocked by the specific antisera was calculated relative to the activity in the presence of the control serum. The actual extent of inhibition of the growth of cells in the presence of the control sera and the following sample concentrations is indicated in parentheses: TGF- β_1 or TGF- β_2 , 2.8 pM; patient 35, 32 µl/ml; patient 4, 4.3 µl/ml; patient 42, 1.8 µl/ml; patient 11, 0.9 µl/ml; serum-free medium conditioned by cultured retinal pigment epithelial cells, 9 µl/ml.

These findings also may shed some light on the source of TGF- β within eyes with PVR. During retinal detachment surgery, various forms of retinopexy are applied to the choroid and retina in order to induce a localized scar in the region of a retinal tear. It has been found that all forms of retinopexy including cryotherapy, photocoagulation, and diatherapy cause significant breakdown of the blood-ocular barriers, thereby allowing serum components access to the intraocular space (31). It has been postulated that these serum components may play a role in inducing the resultant fibrosis (31). In the case of TGF- β , the majority of the growth factor in the intraocular fluid has been found to be type 2 and, therefore, not serum derived. The smaller amount of TGF- β_1 may indeed be derived from serum. This suggests that there may be an intraocular source of TGF- β_2 . We have recently found that the retinal pigment epithelial cells can synthesize and secrete TGF- β_2 in addition to TGF- β_1 in vitro (Connor, T. B., A. B. Roberts, M. B. Sporn, and B. M. Glaser, manuscript in preparation). These findings suggest that the retinal pigment epithelium may play a central role in the development of fibrosis following retinal detachment surgery as has been previously postulated.

Since TGF- β_2 levels are elevated in the vitreous cavity of eyes with increased intraocular fibrosis, we questioned whether intravitreal injections of TGF- β might induce intraocular fibrosis in an animal model. The amount of human TGF- β_2 available to us for this study was limited. However, since no functional differences have yet been found between TGF- β_1 and TGF- β_2 , we have performed these experiments using human TGF- β_1 . We found that TGF- β_1 alone did not induce a significant increase in intravitreal fibrosis when injected into the vitreous cavity. However, TGF- β_1 combined with FN did



result in a significant increase in intravitreal fibrosis and resultant traction retinal detachment while FN alone had no effect. Interestingly, FN has been found in increased levels in human eyes with intraocular fibrosis associated with PVR (32). In addition, TGF- β , but not PDGF nor FGF, has recently been shown to enhance the ability of fibroblasts to contract a collagen matrix in vitro (33). The stimulation of cell-mediated collagen gel contraction was greatly enhanced in the presence of serum (33). It may be that the ability of FN to enhance the ability of intravitreal TGF- β_1 to induce vitreous gel contraction and resultant traction retinal detachment is related to the enhancement of gel contraction in vitro by serum components such as FN. Experiments are currently underway to determine if FN in serum accounts for the latter effect.

Recent studies utilizing both bioassays and immunoassays have failed to demonstrate detectable levels of basic FGF in vitreous aspirates from eyes with PVR or uncomplicated retinal detachments (Snyder, M., T. B. Connor, and B. M. Glaser, manuscript in preparation). Preliminary studies have likewise so far not revealed an association between levels of PDGF in eyes with PVR and the severity of the disease (Grotendorst, G. R., T. B. Connor, and B. M. Glaser, unpublished data).

Thus, in this disease entity, levels of TGF- β_2 are associated with the severity of the fibrotic process, whereas FGF and PDGF may not be similarly associated. The ability of TGF- β to attract fibroblasts and monocytes (6, 7) as well as its unique ability to enhance the synthesis of extracellular matrix components (8, 9) and stimulate cell-mediated contraction of collagen gels may set up a cycle of events that promotes the successive formation of fibrotic tissue in this disease process. The final determination of the role of TGF- β in this disease process awaits the ability to block its activity and assess if this can

retard or arrest fibrosis. The role of TGF- β in fibrosis of other organs and the assessment of the relative roles of TGF- β_1 and TGF- β_2 in these disease processes deserve further investigation.

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